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Interaction of GapA with HPr and Its Homologue, Crh: Novel Levels of Regulation of a Key Step of Glycolysis in *Bacillus subtilis*?▽†

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In *Bacillus subtilis* cells, we identified a new partner of HPr, an enzyme of the glycolysis pathway, the glyceraldehyde-3-phosphate dehydrogenase GapA. We showed that, in vitro, phosphorylated and unphosphorylated forms of HPr and its homologue, Crh, could interact with GapA, but only their seryl-phosphorylated forms were able to inhibit its activity.

The HPr protein of the phosphoenolpyruvate-sugar phosphotransferase system possesses a key regulatory role in several bacteria. Indeed, HPr phosphorylated on the histidyl residue at position 15 by enzyme I regulates the activities of other proteins by phosphorylation-like transcriptional regulators containing the phosphotransferase system regulation domain (19), the glycerol kinase in some *Enterococcus* and *Bacillus* species (1, 4), and the lactose transporter LacS in *Streptococcus thermophilus* (2, 9). In gram-positive bacteria, HPr can also be phosphorylated on the seryl residue at position 46 by an ATP-dependent HPr kinase/phosphorylase, HprK/P (8). In *Lactobacillus casei*, P-Ser-HPr participates in the phenomenon of inducer exclusion (20). By interacting with CcpA, P-Ser-HPr is involved in carbon catabolite regulation (6, 18). In bacilli, HPr possesses a homologue, Crh, which is phosphorylated only by HprK/P at Ser 46. Similarly to P-Ser-HPr, P-Ser-Crh interacts with CcpA and contributes to catabolite repression (6, 7). More recently, it has been shown that P-Ser-HPr can also interact in vitro with another regulator, RbsR of *Bacillus subtilis* (15). In addition, P-Ser-HPr seems to be involved in the virulence mechanism of *Listeria monocytogenes* by inhibiting the transcriptional activator PrfA (11).

In this current study, using tandem affinity purification (TAP), we identified the glyceraldehyde-3-P dehydrogenase GapA (5) as a new interaction partner of HPr. This interaction was confirmed and analyzed by two different biochemical approaches and was also demonstrated for Crh. We then showed that only the seryl-phosphorylated forms of HPr and Crh were able to weakly inhibit GapA activity in vitro.

GapA: a partner for HPr in *B. subtilis* cells? In order to identify new potential functions of HPr in *B. subtilis*, we tried to find new partners of HPr using the TAP method (17). To this end, DNA fragments encoding the HPr protein and the TAG domain (the immunoglobulin G domain, TeV site, and calmodulin binding domain) were generated by PCR using specific primers (see Table S1 in the supplemental material). The TAG domain was amplified using the pBS1479 plasmid (16) as a matrix. The long-flanking-homology PCR protocol (21) was then used to fuse the two fragments, using two primers containing extra nucleotides, allowing an oriented-ligation-independent cloning. The resulting DNA fragment was then inserted into the pDG148-Stu vector (12). The *B. subtilis* cells transformed by the pDG148-TAP-HPr plasmid were grown at 37°C in LB medium containing 1% glucose. The expression of the HPr fused at its C terminus with the TAP tag was induced for 90 min with 0.05 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Then, TAP was carried out as described by Puig et al. (16), by two steps of protein purification using IgG beads and then calmodulin resin. Purified proteins were TCA precipitated and loaded on a 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel stained with the Gel Code Blue stain reagent from Pierce (Fig. 1). Then, the bands were extracted from the gel and the proteins were identified by matrix-assisted laser desorption ionization peptide mapping. Among these proteins, we identified several contaminants from the ribosomal protein family (L3, L6, L11, S4, S5, and S8), often purified by TAP; proteins involved in different cell functions (DNA gyrase, GroEL, elongation factor-TU, and SpoOM, etc.); and the glyceraldehyde-3-P dehydrogenase GapA (5). As a positive control for our TAP approach, we have previously detected the well-characterized HPr-HprK/P interaction (data not shown). However, no *B. subtilis* regulatory soluble proteins known to interact with HPr, e.g., LevR, LicT, and CcpA, were purified and identified by this technique. This could be due either to the low level of expres-

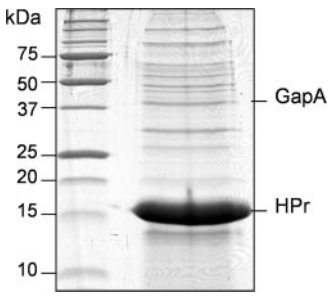

FIG. 1. Analysis of affinity-purified complexes. Results are shown for SDS-PAGE of TAP-tagged HPr copurified proteins obtained from the *B. subtilis* 168 strain and grown on LB medium supplemented with 1% glucose.

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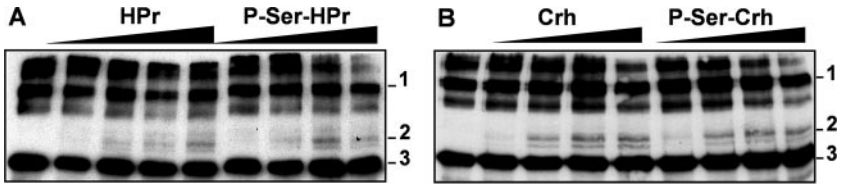

FIG. 2. Western blot analysis of GapA cross-linked with HPr, Crh, P-Ser-HPr, and P-Ser-Crh. (A) HPr and P-Ser-HPr proteins. (B) Crh and P-Ser-Crh proteins. For each interaction, increasing amounts of interactant (0, 0.1, 0.5, 1, and 2 μM) were used for a constant amount of GapA (0.5 μM) in phosphate-buffered saline (25-μl final volume) and preincubated for 5 min at room temperature. Disuccinimidyl glutarate (50 μM) was then added to the protein mixture and incubated for 25 min at room temperature. The reaction was stopped by addition of 5 μl of Laemmli denaturing solution and loaded on a 10% SDS-PAGE gel. Proteins were then transferred on nitrocellulose membrane and detected by Western blotting with anti-T7 tag HRP-conjugated antibodies (1/100,000) from Novagen.

sion of these proteins or to the strength of the interaction, which is too weak to resist the two steps of purification. Indeed, P-Ser-HPr was not retained on a CcpA column but eluted only with a delay in comparison to nonphosphorylated HPr (3).

GapA interacts with phosphorylated and unphosphorylated forms of HPr and Crh in vitro. In order to validate the interaction of GapA with HPr, biochemical experiments were performed. For this purpose, using specific primers (see Table S1 in the supplemental material), the *gapA* gene was amplified and cloned in the pET21a plasmid. Then, a GapA protein fused to a six-histidine and a T7 tag was produced, purified on Ni-nitrilotriacetic acid resin (7), and incubated in the presence of purified HPr (Fig. 2A) or Crh (Fig. 2B) and the cross-linker disuccinimidyl glutarate. The mixed proteins were loaded on an SDS-PAGE gel, separated, transferred to a nitrocellulose membrane, and finally detected by Western blot analysis using anti-T7 tag horseradish peroxidase (HRP)-conjugated antibodies. Only GapA was detected with the antibody since neither HPr nor Crh was tagged with the T7 tag. In all the lanes shown in Fig. 2, we can observe several bands corresponding to the different oligomeric forms of GapA: band 1 migrates at around 150 kDa and corresponds to the tetrameric active form, whereas band 3 migrates at around 37 kDa and corresponds to a monomer. A band (Fig. 2, band 2) appeared with increasing amounts of added HPr or Crh. It migrates slightly below the band of 50 kDa of our standard and corresponds to the size of one monomer of GapA (36 kDa) plus one monomer of HPr or Crh (9 kDa). The same experiment was performed with the stable P-Ser forms of the proteins (Fig. 2) and gave the same results, i.e., HPr, Crh, P-Ser-HPr, and P-Ser-Crh were all able to bind to GapA. However, using anti-T7 tag HRP-conjugated antibodies, it was not possible to detect the most probable

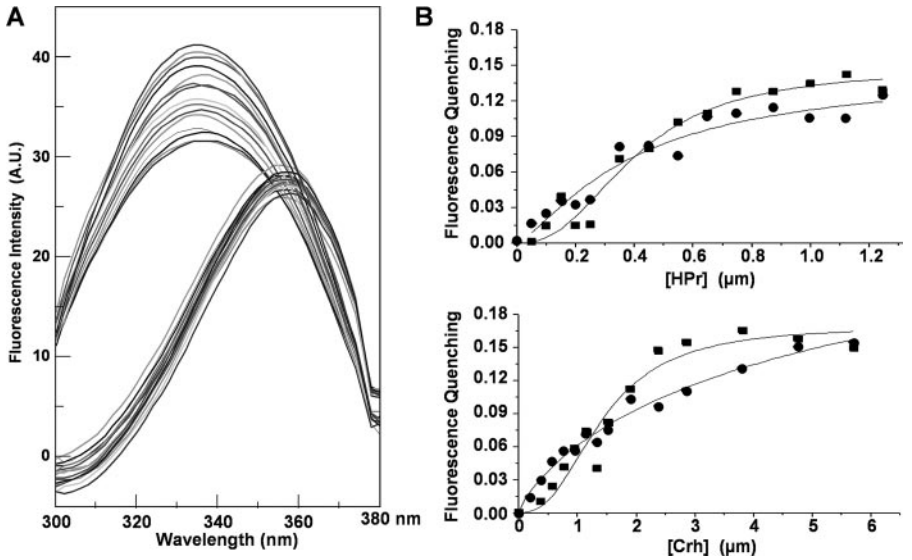

FIG. 3. Effect of HPr, P-Ser-HPr, Crh, or P-Ser-Crh on GapA tryptophan fluorescence. Fluorescence measurements were carried out after dilution of GapA (1 μM final concentration) and equilibration for 2 min in 2 ml of 25 mM HEPES-KOH buffer, pH 8, at 25°C. Increasing concentrations of HPr, P-Ser-HPr, Crh, or P-Ser-Crh were then added, and the emission fluorescence was scanned in the range of 300 to 380 nm, upon excitation at 282 nm. Corrections for the inner-filter effect of the ligands were performed under the same conditions by using *N*-acetyltryptophanamide (NATA). (A) Effect of P-Ser-Crh on the GapA fluorescence spectrum. All spectra were corrected for buffer fluorescence. From the upper to the lower curves, the concentrations of P-Ser-Crh were 0, 0.19, 0.38, 0.57, 0.76, 0.95, 1.14, 1.33, 1.52, 1.90, 2.37, 2.85, 3.8, 4.75, and 5.7 μM, respectively. The GapA fluorescence spectrum is centered at 335 nm and the NATA spectrum at 357 nm. (B) The quenching of GapA fluorescence was plotted versus the concentration of HPr or Crh after correction for the inner-filter effect. Peak integration was carried out for each ligand concentration. The enzymatic curves were realized in triplicate for each protein, and curve fitting for the data was performed by using Microcal Origin 5.0 software. The black circles (●) represent the phosphorylated proteins, and the black squares (■) represent the nonphosphorylated proteins.

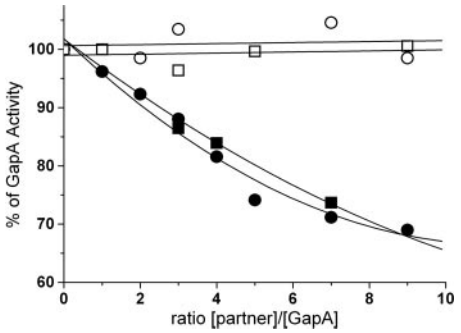

FIG. 4. Effects of HPr, P-Ser-HPr, Crh, and P-Ser-Crh on the activity of GapA. A 1-ml reaction mixture containing 40 mM ethanolamine, pH 8.0, 50 mM Na_2HPO_4, 0.2 mM EDTA, 2 mM β-NAD hydrate (β-NAD^+), and 1.5 μM GapA with either HPr, Crh, P-Ser-HPr, or P-Ser-Crh (0 to 9 μM) was incubated for 5 min at 25°C before addition of 2 mM D,L-glyceraldehyde-3-phosphate. GapA activity was monitored following the reduction of β-NAD^+ at 340 nm. Results were expressed percentages of GapA residual activities, determined by calculation of the slope of the β-NAD^+ hydrolysis. The percentage of GapA residual activity was then reported for each ratio of added partner and GapA concentration. For each protein, HPr (□), Crh (○), P-Ser-HPr (■), and P-Ser-Crh (●), the curve is an average for experiments realized at least in triplicate.

interaction with the tetramer in this 10% SDS-PAGE gel since we cannot discriminate between 144 kDa (for the GapA tetramer) and 184 kDa (for the GapA tetramer in interaction with four molecules of HPr or Crh). We then performed Western blot analysis using anti-HPr antibodies. In these conditions, the addition of GapA induced the apparition of a retarded band of around 200 kDa, which probably corresponds to four molecules of HPr in interaction with the GapA tetramer (data not shown).

To confirm these interactions, we decided to record the intrinsic fluorescence of GapA in the presence of increasing concentrations of HPr, Crh, P-Ser-HPr, or P-Ser-Crh. Indeed, GapA possesses two tryptophans (Trp 84 and Trp 311) and neither HPr nor Crh possesses any Trp. We also used the *B. subtilis* ClpY protein not possessing any tryptophan as a negative control. For each addition of interactant, except for the negative control, we observed a strong quenching of fluorescence for GapA (results for the addition of P-Ser-Crh are shown in Fig. 3A). We therefore conclude that the binding of HPr or Crh induces a conformational change in GapA. We can note that, under our experimental conditions, HPr possesses an affinity three times higher for GapA than for Crh (K_d values of 0.40 ± 0.05 μM and 1.5 ± 0.2 μM, respectively). We also observed that the shapes of the binding curves were different for the phosphorylated and nonphosphorylated forms of HPr and Crh, suggesting differences in their binding to GapA (Fig. 3B).

GapA is not phosphorylated by P-His-HPr. HPr was already shown to regulate enzyme activity. However, this regulation was carried out by P-His-HPr, which phosphorylates a histidyl residue of the glycerol kinase (1). Since *B. subtilis* GapA shares some highly conserved histidines with GapA from other bacteria (His 108 and His 178) and interacts with HPr, we tested whether GapA could be phosphorylated by P-His-HPr. However, no histidyl phosphorylation of GapA was detected (see Fig. S1 in the supplemental material).

P-Ser-HPr and P-Ser-Crh inhibit specifically GapA activity in vitro. In order to understand the role of these interactions, we measured the GapA activity in the presence of HPr, Crh, or the phosphorylated forms of these proteins. No inhibition of GapA activity was detected in the presence of HPr or Crh (Fig. 4) or in the presence of P-His-HPr (data not shown). By contrast, the presence of the seryl-phosphorylated proteins induced an inhibition of GapA activity. Furthermore, the levels of inhibition, around 35%, are similar for P-Ser-HPr and P-Ser-Crh despite the fact that these two proteins do not have the same affinity for GapA (Fig. 3B). These observations suggest that our in vitro conditions were not optimal and maybe another cofactor would be necessary to observe a better inhibition. This result is surprising since transcriptomic and proteomic studies (10, 13, 14) have shown that *gapA* expression or GapA synthesis was induced by glucose and that an *hprK* mutation abolished the glucose effect on *gapA* operon expression (13). As a control, we tested whether the phosphorylated or unphosphorylated HPr and Crh proteins were also able to inhibit GapB, a highly homologous GapA protein which catalyzes the reverse reaction in gluconeogenesis (5). However, no inhibition of GapB activity was observed (data not shown).

In conclusion, we identified by the TAP method a new partner for the HPr protein, GapA, a key enzyme of the glycolysis pathway. This interaction was demonstrated in vitro, and our results suggest a new regulatory function for seryl-phosphorylated HPr and Crh, which has to be confirmed in vivo.

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